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Molecular phylogeny and species delimitation in the section *Longibrachiatum* of *Trichoderma*

Irina S. Druzhinina [a,*], Monika Komoń-Zelazowska [b,1], Adnan Ismaiel [c,1], Walter Jaklitsch [d], Temesgen Mullaw [a,2], Gary J. Samuels [c], Christian P. Kubicek [a]

[a] Research Area Biotechnology and Microbiology, Institute of Chemical Engineering, Vienna University of Technology, Getreidemarkt 9/166-5, A-1060 Vienna, Austria
[b] Austrian Institute of Industrial Biotechnology (ACIB), GmBH c/o Institute of Chemical Engineering, Vienna University of Technology, Gumpendorferstraße 1a, A-1060 Vienna, Austria
[c] United States Department of Agriculture, ARS, Systematic Mycology & Microbiology Laboratory, B-010a, Rm 213, 10300 Baltimore Ave., Beltsville, MD 20705, USA
[d] Faculty Centre of Biodiversity, University of Vienna, Rennweg 14, A-1030 Vienna, Austria

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ABSTRACT

The phylogenetically most derived group of the genus *Trichoderma* – section *Longibrachiatum*, includes some of the most intensively studied species, such as the industrial cellulase producer *T. reesei* (teleomorph *Hypocrea jecorina*), or the facultative opportunistic human pathogens *T. longibrachiatum* and *H. orientalis*. At the same time, the phylogeny of this clade is only poorly understood. Here we used a collection of 112 strains representing all currently recognized species and isolates that were tentatively identified as members of the group, to analyze species diversity and molecular evolution. Bayesian phylogenetic analyses based on several unlinked loci in individual and concatenated datasets confirmed 13 previously described species and 3 previously recognized phylogenetic species all of which were not yet described formally. When the genealogical concordance criterion, the *K/θ* method and comparison of frequencies of pairwise nucleotide differences were applied to the data sample, 10 additional new phylogenetic species were recognized, seven of which consisted only of a single lineage. Our analysis thus identifies 26 putative species in section *Longibrachiatum*, what doubles the currently estimated taxonomic diversity of the group, and illustrates the power of combining genealogical concordance and population genetic analysis for dissecting species in a recently diverged group of fungal species.

1. Introduction

Species of the mycotrophic filamentous ascomyceteous genus *Trichoderma* (Hypocreales, Hypocreaceae; teleomorph *Hypocrea*) are among the most commonly encountered fungi (Druzhinina et al., 2011). They are frequently isolated from soil and are found growing on dead wood, bark, other fungi, building materials and animals, including humans, demonstrating a high opportunistic potential and adaptability to ecological conditions (Klein and Eveleigh, 1998; Druzhinina et al., 2011). Taxonomically, *Trichoderma* had been divided into five sections, including section *Longibrachiatum* (for review see Gams and Bissett, 1998), but with increasing molecular phylogenetic analyses the sectional nomenclature of *Trichoderma* was abandoned in favor of naming phylogenetic clades (Samuels, 2006; Kubicek et al., 2008). Interestingly, though, the morphologically and metabolically distinctive section *Longibrachiatum* is one of only two sections that has remained intact following phylogenetic analysis. The comparative analysis of three genomes of diverse *Trichoderma* species has revealed that the *Longibrachiatum* clade is evolutionarily one of the youngest clades (Kubicek et al., 2011) of the genus. Sexual reproduction is common in the *Longibrachiatum* clade: Samuels et al. (1998) defined 10 species within what they called the *'Hypocrea schweinitzii* complex'.

The *Longibrachiatum* clade comprises the most intensively studied *Trichoderma* species, *T. reesei* (teleomorph *Hypocrea jecorina*), which is industrially used for the production of cellulolytic and hemicellulolytic enzymes involved in food and feed industry, textile manufacture and biofuel technology (Harman and Kubicek, 1998; Kubicek et al., 2009). In addition, several members of the clade are used for production of secondary metabolites, particularly strains that were isolated from marine habitats (Sperry et al., 1998; Ruiz et al., 2007; Paz et al., 2009; Gal-Hamed et al., 2011). However, certain strains of three of its species, *T. citrinoviride* (teleomorph *H. schweinitzii*), *T. longibrachiatum* and *H. orientalis*, have caused opportunistic infections of immunocompromized humans (Kuhls et al., 1999; Kredics et al., 2003), and *T. longibrachiatum* and *T. citrinoviride*

* Corresponding author. Address: Microbiology Group, Research Area Biotechnology and Microbiology, Institute of Chemical Engineering, Vienna University of Technology, Gumpendorferstrasse 1a, A-1060 Vienna, Austria. Fax: +43 1 58801 17299.
E-mail address: druzhini@mail.zserv.tuwien.ac.at (I.S. Druzhinina).
[1] These authors contributed equally to the research.
[2] Present address: Center for Environmental Science, Institute of Marine and Environmental Technology, University of Maryland, Baltimore, MD, USA.

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are frequently isolated as indoor contaminants with high allergenic potential for humans (Thrane et al., 2001).

Species delimitation in fungi is still a matter of intensive debate, and several species concepts have been discussed (for review see Giraud et al., 2008). The first molecular phylogenetic analysis of the *Longibrachiatum* clade (Kuhls et al., 1997) was based on the internal transcribed spacer region of the rRNA gene cluster (ITS). Although this region is currently considered to be a universal barcode locus for fungi (Bellemain et al., 2010), it is unable to distinguish all closely related species in many genera of hyphomycetes including *Trichoderma* (Gazis et al., in press). Today, phylogenetic species concept has become most popular, because it bypasses the limitations imposed by the morphological or biological species concepts (such as the requirement for clear phenotypic differences, or the ability to mate the fungus *in vitro*), and because of the simplicity with which gene sequences can be obtained from practically all organisms. Thereby, the GCPSR (Genealogical Concordance Phylogenetic Species Recognition, Taylor et al., 2000) concept, which uses the phylogenetic concordance of multiple unlinked genes to identify the absence of genetic exchange and thus evolutionary independence of lineages, is currently most widely used within the fungal kingdom (e.g. Dettman et al., 2003; Fournier et al., 2005; Johnson et al., 2005; Koufopanou et al., 2001; Le Gac et al., 2007; Pringle et al., 2005). The molecular phylogeny of some species of the *Longibrachiatum* clade was investigated recently using GCPSR (Druzhinina et al., 2008, 2010; Atanasova et al., 2010) with the result that some of the taxa in fact comprised clonal species (or agamospecies) that reproduce exclusively asexually. Druzhinina et al. (2008, 2010) therefore hypothesized that the loss of sexual reproduction may constitute an important mechanism for speciation in the *Longibrachiatum* clade. Yet, whether or not a lineage is indeed a phylogenetic species or e.g. represents demes from a metapopulation that is connected by infrequent migration, can be obscured. In addition, GCPSR can be difficult to apply to truly clonal fungi where no incongruities in multi-locus data are found.

Birky et al. (2010) recently developed a population genetics approach, which can be used to complement species recognition by GCPSR. Their method is based on the theory that in a single species random genetic drift will produce clades and singlets that have all descended from a common ancestor on an average $2N_e$ generations ago (N_e is the effective population size), and their distance from each other will be less than $2N_e$ generations. After the onset of speciation, however, a species will be split into two populations that are completely separated and will thus form clusters separated by a gap exceeding $2N_e$. Thus clusters that are separated by $t \geqslant 4N_e$ generations (the "4× rule" or "K/θ method") represent the upper 95% confidence limit of the coalescent time, and are characterized by a probability of less than 5% of those being formed by random genetic drift. The K/θ method therefore supports the cluster as an evolutionary species (Birky et al., 2010).

Since the earlier systematic work on the *Longibrachiatum* clade (Bissett, 1984; Kuhls et al., 1997; Samuels et al., 1998) we have received numerous cultures that are members of the clade that cannot be molecularly identified with certainty as any of the recognized species. This uncertainty, combined with the discovery of cryptic species in the clade through the use of GCPSR has leaded us to apply the GCPSR concept and the K/θ method to the enlarged collection of isolates of the *Longibrachiatum* clade.

2. Materials and methods

2.1. Material studied

Fungal strains were independently received by the Vienna University of Technology and USDA labs from colleagues in several research institutions or from personal collections. Most *Trichoderma* cultures were obtained by direct isolation from the substratum. Several collections were derived from stromata of *Hypocrea* teleomorphs. Pure cultures were made by isolating single ascospores or conidia using a micromanipulator or a platinum needle on cornmeal agar (Difco) + 2% (w/v) dextrose (CMD). The strains, their origins and the NCBI GenBank accession numbers of DNA sequences used in this work are listed in Table 1. The isolates are stored at −80 °C in 20–50% glycerol in the laboratory of Vienna University of Technology (Austria) or at the USDA (Beltsville, MD, USA) or the University of Vienna (Austria). Representative strains are deposited in the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands (CBS).

2.2. DNA extraction, PCR amplification and sequencing

Mycelia were harvested after 2–4 days of growth on 3% malt extract agar (MEA) or up to 7 d in liquid 2% malt extract medium at 25 °C and genomic DNA was isolated using QIAGEN DNeasy® Plant Mini Kit following the manufacturer's protocol. Amplification of fragments of *tef1* (translation elongation factor 1-α), *cal1* (calmodulin), *chi18-5* (endochitinase CHI18-5, former known as *ech42*) and of *rpb2* (RNA polymerase subunit B II) was performed as described previously (Druzhinina et al., 2008, 2010; Atanasova et al., 2010). PCR fragments were purified (PCR purification kit, Qiagen, Hilden, Germany), and sequenced at MWG (Ebersberg, Germany) or cycle-sequenced the University of Vienna after an *in vitro* enzymatic cleanup (Werle et al., 1994). In Beltsville, sequences were obtained using BigDye Terminator cycle sequencing kit v. 3.1 (Applied Biosystems, Foster City, CA, USA), and products were analyzed directly on a 3130 Genetic Analyzer (Applied Biosystems). For each locus both strands were sequenced with the primers used in PCR amplifications.

2.3. Phylogenetic analysis

For the phylogenetic analysis DNA sequences were aligned with Clustal X 1.81 (Thompson et al., 1997) and then visually checked in GeneDoc 2.6 (Nicholas and Nicholas, 1997). Optionally ambiguous areas of the alignment were removed using the gblocks server http://molevol.cmima.csic.es/castresana/Gblocks_server.html (Castresana, 2000). The loci used in this study were previously checked for absence of intragenic recombination (Druzhinina et al., 2008). Neutral evolution was tested by linkage disequilibrium based statistics and Tajima's test as implemented in DnaSP 4.50.3 (Rozas et al., 2003). The interleaved NEXUS file was formatted using PAUP* 4.0b10 (Swofford, 2002). The best nucleotide substitution model for each locus was determined using jMODELTEST (Posada, 2003) and the unconstrained GTR + I + G nucleotide substitution model was applied to all loci. Metropolis-coupled Markov chain Monte Carlo (MCMCMC) sampling was performed using MrBayes v. 3.0B4 with two simultaneous runs of four incrementally heated chains that performed for 5 millions of generations. The sufficient number of generations for each dataset was determined using the AWTY graphical system (Nylander et al., 2008) to check for convergence of MCMCMC. Bayesian posterior probabilities (PP) were obtained from the 50% majority-rule consensus of trees sampled every 100 generations after removing the first trees. PP values lower than 0.95 were not considered significant while values below 0.9 are not shown on the resulting phylograms. Model parameters summaries after MCMCMC run and burning first samplings as well as nucleotide characteristics of used loci are given in Table 2.

Table 1
Strains used and NCBI GenBank accession numbers.

Taxon	Isolate number	Other numbers	Origin	Recognized as a species in	Published in	NCBI GeneBank accession numbers			
						tef1	*cal1*	*chi18–5*	*rpb2*
Formally described species									
H. andinensis	**G.J.S. 90-140**	CBS 354.97, ATCC 208857	Venezuela	Samuels et al. (1998)	Samuels et al. (1998)	AY956321	JN175412	JN175472	JN175531
H. novae-zelandiae	**G.J.S. 81-265**	CBS 639.92, CBS 496.97, ATCC 28856	New Zealand	Samuels et al. (1998)	Samuels et al. (1998)	AY937448	JN175406	JN175465	DQ641672
	G.J.S. 81-264	CBS 472.97	New Zealand		Samuels et al. (1998)	JQ513358	JN175407	JN175466	DQ641672
	G.J.S. 99-113		New Zealand			JN175582	JN175408	JN175467	JN175526
H. orientalis	G.J.S. 04-321		Peru			JN175573	JN175397	JN175455	JN175517
	G.J.S. 04-332		Peru			JN175574	JN175398	JN175456	JN175518
	G.J.S. 04-333		Peru			JN175575	JN175399	JN175457	JN175519
	G.J.S. 04-316		Peru			JN175576	JN175400	JN175458	JN175520
	DIS 270f		Ecuador			JN175577	JN175401	JN175459	JN175521
	G.J.S. 09-784		Peru			JN175578	JN175402	JN175460	JN175522
	G.J.S. 10-230		Brazil			JN175579	JN175403	JN175461	JN175523
	G.J.S. 88-81		China	Samuels et al. (1998) Druzhinina et al. (2008)	Druzhinina et al. (2008)	EU401581	EU401448	EU401500	n/a
	G.J.S. 91-157		Germany		Druzhinina et al. (2008)	EU401609	EU401693	EU401461	EU401513
	CECT 2606		Sierra Leone		Samuels et al. (1998)	EU401609	EU401477	EU401528	n/a
	C.P.K. 688	TUB F-837	Costa Rica		Druzhinina et al. (2005)	AY857282	EU401452	EU401504	n/a
	C.P.K. 683	TUB-F 831	Costa Rica		Druzhinina et al. (2005)	EU401584	EU401451	EU401503	n/a
	C.P.K. 704	TUB F-1023	Argentina		Druzhinina et al. (2005)	EU401585	EU401453	EU401505	n/a
	G.J.S. 10-253		Tanzania			JN544898	JN388899	JN544899	n/a
	PPRI 3894		South Africa		Druzhinina et al. (2008)	EU401579	EU401446	EU401498	n/a
	UAMH 9573		Canada		Druzhinina et al. (2008)	EU401599	EU401467	EU401519	n/a
H. schweinitzii/T. citrinoviride	**CTR 79-225**		USA		Samuels et al. (1998)	JN175590	JN175418	JN175478	JN175537
	G.J.S. 90-111		USA		Samuels et al. (1998)	JN175591	JN175419	JN175479	JN175538
	CTR 79-290		USA		Samuels et al. (1998)	JN175592	JN175420	JN175480	JN175539
	DAOM 145647		USA		Samuels et al. (1998)	AY937422	JN175421	JN175481	JN175540
	TR 106		USA		Samuels et al. (1998)	JN175593	JN175422	JN175482	JN175541
	G.J.S. 01-18		Russia			JN175594	JN175423	JN175483	JN175542
	DAOM 139758	DAOM 139758	Canada	Samuels et al. (1998)	Samuels et al. (1998)	EU338334	JQ389878	JN175484	JN175543
	G.J.S. 92-8	CBS 636.92, IMI 352472	France		Samuels et al. (1998)	JN175595	JN175424	JN175485	JN175544
	TR 102		USA		Samuels et al. (1998)	JN175596	JN175425	JN175486	JN175545
T. effusum	C.P.K. 254	DAOM230007	India	Bissett et al. (2003)	Bissett et al. (2003)	JN182272	JN182286	JN182295	JQ513368
T. ghanense	ATCC 28019		USA		Samuels et al. (1998)	JN175606	JN175435	JN175496	JN175555
	G.J.S. 07-29		Ghana			JN175607	JN175436	JN175497	JN175556
	G.J.S. 07-28		Ghana			JN175608	JN175437	JN175498	JN175557
	G.J.S. 06-157		Nigeria			JN175609	JN175438	JN175499	JN175558
	G.J.S. 08-208		USA			JN133556	JN133530	JN175500	JN133562
	G.J.S. 95-137	IAM 13109	Ghana	Samuels et al. (1998)	Samuels et al. (1998)	AY937423	JN175439	JN175501	JN175559
	DAOM 165776				Samuels et al. (1998)	JN175610	JN175440	JN175502	JN175560
	G.J.S. 08-114		Argentina			JN175611	JN175441	JN175503	JN175561
	G.J.S. 04-313		Peru			JN175612	JN175442	JN175504	JN175562
	G.J.S. 04-323		Peru			JN175613	JN175443	JN175505	JN175563
	C.P.K. 2057		Hungary		Hatvani et al. (2007)	JN182282	JN182292	JN182307	JN182314
	G.J.S. 05-96		Italy			JN175614	JN175444	JN175506	HQ260617
T. konilangbra	C.P.K. 132		Uganda	Samuels et al. (1998)	Samuels et al. (1998)	JN258681	JN182285	JN182300	JQ513367
	C.P.K. 133		Uganda		Samuels et al. (1998)	JQ513357	JQ513346	JQ513361	n/a
T. longibrachiatum	ATCC 18648		USA	Samuels et al. (1998) Druzhinina et al. (2008)	Samuels et al. (1998)	EU401591	EU401459	EU401511	DQ087242

	G.J.S. 01-121		Netherlands			JN175564	JN175387	JN175445	JN175507
	G.J.S. 08-198		Brazil			JN175565	JN175388	JN175446	JN175508
	G.J.S. 04-31	CGS 118640 , ATCC MYA-3642	Mexico			DQ297069	JN175389	JN175447	JN175509
	G.J.S. 08-104		Argentina			JN175566	JN175390	JN175448	JN175510
	G.J.S. 04-101		Vietnam			JN175567	JN175391	JN175449	JN175511
	G.J.S. 04-53		Vietnam			JN175568	JN175392	JN175450	JN175512
	G.J.S. 07-21		Ghana			JN175569	JN175393	JN175451	JN175513
	G.J.S. 08-119		Argentina			JN175570	JN175394	JN175452	JN175514
	C.P.K. 1707		Russia			EU401610	EU401478	EU401529	JN182315
	C.P.K. 842	CBS 115338	Egypt		Wuczkowski et al. (2003)	EU401587	EU401455	EU401507	JN182316
	C.P.K. 744	TUB F-1237[a]	Fiji			JN182276	JN182288	n/a	JN182308
T. parareesei	G.J.S. 04-41		Brazil		Druzhinina et al. (2010)	GQ354372	GQ354306	HM182989	HM182964
	G.J.S. 07-26		Ghana		Druzhinina et al. (2010)	GQ354373	GQ354307	HM182991	HM182966
	C.P.K. 634	TUB F-430	Sri Lanka		Druzhinina et al. (2010)	GQ354351	GQ354285	HM182993	HM182968
	C.P.K. 717	TUB F-1066	Argentina	Atanasova et al. (2010), Druzhinina et al. (2010)	Druzhinina et al. (2010)	GQ354353	GQ354288	HM182987	HM182963
	C.P.K. 523	TUB F-1034	Taiwan	Druzhinina et al. (2010)	Kubicek et al. (2003)	GQ354349	GQ354283	HM183006	HM182981
	C.P.K. 524	TUB F-1038	Taiwan	Druzhinina et al. (2010)	Kubicek et al. (2003)	GQ354350	GQ354284	HM183007	HM182982
	G.J.S. 04-93		Vietnam			JN175605	JN175434	JN175495	JN175554
T. pseudokoningii	**G.J.S. 81-300**	CBS 254.97, CBS 432.97	New Zealand		Samuels et al. (1998)	AY937429	JN175415	HM183010	HM182985
	NS 19	DAOM 167678, CBS 480.91, ATCC 298861	Australia		Samuels et al. (1998)	JN175588	JN175416	JN175476	JN175535
	G.J.S. 99-149		Australia			JN175589	JN175417	JN175477	JN175536
H. jecorina/T. reesei	**G.J.S. 00-89**		Brazil			JN175599	JN175428	JN175489	JN175548
	G.J.S. 00-09		Mexico			JN175600	JN175429	JN175490	JN175549
	G.J.S. 93-22	ATCC 208850	New Caledonia		Samuels et al. (1998)	GQ354363	GQ354297	HM183001	HM182276
	G.J.S. 09-74		Peru			JN175601	JN175430	JN175491	JN175550
	G.J.S. 06-138		Cameroun		Druzhinina et al. (2010)	GQ354370	GQ354304	HM182997	HM182972
	QM 6a	NS 20	Solomon Islands	Samuels et al. (1998) Druzhinina et al. (2010)	Samuels et al. (1998)	Z23012	JN180917	HM182994	HM182969
	G.J.S. 93-23		New Caledonia		Samuels et al. (1998)	GQ354363	GQ354290	HM183000	HM182975
	G.J.S. 10-189		India			JN175602	JN175431	JN175492	JN175551
	G.J.S. 97-38	CBS 999.97 , ATCC 204423	French Guiana		Lieckfeldt et al. (2000)	JN175603	JN175432	JN175493	JN175552
	G.J.S. 04-115		Vietnam			JN175604	JN175433	JN175494	JN175553
	G.J.S. 06-140		Cameroun		Druzhinina et al. (2010)	GQ354371	GQ354305	HM189226	HM189271
T. saturnisporum	CBS 335.92		Italy		Samuels et al. (1998)	JN182279	JN182291	JN182296	n/a
	CBS 886.72		South Africa		Samuels et al. (1998)	JN182280	JN388898	JN182297	n/a
	ATCC 28023		USA		Samuels et al. (1998)	JN388897	JN180915	JN175462	JN175524
	ATCC 18903		USA		Samuels et al. (1998)	JN182278	JN182290	JN182298	JN182309
	C.P.K. 3406		Dominican Republic			JN258682	JN258683	JN258687	JN258690
T. sinense	DAOM 230004		Taiwan		Bissett et al. (2003)	AY750889	JN175410	JN175469	JN175528
	C.P.K. 530		Taiwan		Bissett et al. (2003)	JN182273	JQ513347	JN182301	JN182310
	C.P.K. 531		Taiwan		Bissett et al. (2003)	JN182274	JQ513348	JN182302	JN182311
Previously recognized phylogenetic species and lone lineages									
H. sp. CBS 243.63	**CBS 243.63**		New Zealand	Druzhinina et al. (2008)	Samuels et al. (1998)	EU401592	EU401460	EU401512	JQ513369
T. sp. PS III	C.P.K. 1817		Ethiopia	Druzhinina et al. (2008)	Mullaw et al. (2010)	EU401614	EU401482	EU401533	n/a
	C.P.K. 1837		Ethiopia	Druzhinina et al. (2008)	Mullaw et al. (2010)	EU401615	EU401483	EU401534	HM182986
	C.P.K. 1841		Ethiopia	Druzhinina et al. (2008)	Mullaw et al. (2010)	EU401616	EU401484	EU401535	n/a
T. sp. C.P.K. 3334	C.P.K. 3503		Ethiopia	Mullaw et al. (2010)		FJ763179	JQ513349	JQ513362	n/a
	C.P.K. 3524		Ethiopia	Mullaw et al. (2010)		FJ763183	JQ513352	JQ513365	n/a

(*continued on next page*)

Table 1 (*continued*)

Taxon	Isolate number	Other numbers	Origin	Recognized as a species in	Published in	NCBI GeneBank accession numbers			
						tef1	*cal1*	*chi18–5*	*rpb2*
	C.P.K. 3522		Ethiopia	Mullaw et al. (2010)		JQ513359	JQ513350	JQ513363	n/a
	C.P.K. 3523		Ethiopia	Mullaw et al. (2010)		JQ513360	JQ513351	JQ513364	n/a
	C.P.K. 3525		Ethiopia	Mullaw et al. (2010)		FJ763184	JQ513353	JQ513366	n/a
	C.P.K. 3334		Ethiopia	Mullaw et al. (2010)		FJ763149	JQ513354	JN258684	JN258688
	C.P.K. 3350		Ethiopia	Mullaw et al. (2010)		FJ763163	JQ513356	JN258686	n/a
	C.P.K. 3345		Ethiopia	Mullaw et al. (2010)		FJ763158	JQ513355	JN258685	JN258689
Phylogenetic species and lone lineages discovered in this study									
T. sp. MA 3642	G.J.S. 99-3	ATCC 20898	Japan			JN175584	JN175411	JN175470	JN175529
	C.P.K. 885	MA 3642	Austria		Wuczkovsky et al. (2003)	JN182277	JN182289	JN182303	n/a
	G.J.S. 06-66		Vietnam			JN175585	n/a	JN175471	JN175530
	C.P.K. 2883		Hungary		Hatvani et al. (2007)	JN182283	JN182293	JN182304	JN182312
	C.P.K. 3412		Taiwan			JN182284	JN182294	JN182305	n/a
H. sp. nov. G.J.S. 02-120	**G.J.S. 04-100**		Vietnam			JN175571	JN175395	JN175453	JN175515
	G.J.S. 02-120		Sri Lanka			JN175572	JN175396	JN175454	JN175516
T. sp. nov. TR175	S19		Italy			JN175580	JN175404	JN175463	
	TR 175		USA			JN182281	JQ349444	JN182299	DQ857348
T. sp. nov. G.J.S. 99-17	G.J.S. 99-17		Japan			JN175581	JN175405	JN175464	JN175525
T. sp. nov. G.J.S. 00-72	G.J.S. 00-72		Reunion			JN175583	JN175409	JN175468	JN175527
T. sp. nov. G.J.S. 10-263	G.J.S. 10-263	TUB 2543	Malaysia			JN175598	JN175427	JN175488	JN175547
T. sp. nov. G.J.S. 08-81	G.J.S. 08-81		Mexico			JN175597	JN175426	JN175487	JN175546
T. sp. nov. G.J.S. 01-355	G.J.S. 01-355		Saudi Arabia			JN175586	JN175413	JN175473	JN175532
T. sp. nov. G.J.S. 09-62	G.J.S. 09-62		Peru			JN175587	JN175414	JN175474	JN175533
T. sp. nov. C.P.K. 667	C.P.K. 667	TUB F-739[a]	USA			JN182275	JN182287	JN182306	JN182313

[a] These strains were obtained from G. Szakacs in the course of a bilateral project between Vienna University of Technology and Budapest University of Technology and Economics; type strains for formally described species are underlined; strains isolated from teleomorphs are given in bold.

2.4. Detection of phylogenetic species

We used three approaches to identify phylogenetic species within our sample. The first was the Genealogical Concordance Phylogenetic Species Recognition concept (GCPSR, Taylor et al., 2000), which identifies a phylogenetic species from the existence of statistically supported phylogenetic clades that are present in the majority (at least two of three) of single-locus trees and that are not contradicted by any other single-gene tree(s) determined by the same method. To identify such clades, we used the approach of Dettman et al. (2003), i.e. production and analysis of a majority-rule consensus tree from the three single-locus trees, which reveals the genealogical patterns shared among loci, regardless of levels of support.

The second criterion was the K/θ method (Birky et al., 2010). Briefly, this involves: (i) estimation of the nucleotide diversity π (using DnaSp v5.0; Rozas et al., 2003) by the mean pairwise difference between sequences multiplied by the sample size correction $n/(n-1)$ where n is the number of sequences in the clade; (ii) calculating θ ($\approx 2N_e\mu$) from $\pi/(1–4\pi/3)$; (iii) testing the nucleotide diversity K between each pair of sister clades; and (iv) calculation of K/θ, which consequently should be >4 in the case of a true evolutionary species.

Third, we compared the frequency of pairwise nucleotide sequence differences within our sample. As shown by Highton (2000), this procedure will result in a bimodal frequency distribution of pairwise sequence differences, among which the lower values represent sequence differences between individuals within species, with an expected mean of $N_e\mu 2N_e\mu$ differences per site, whereas the second mode represents differences between species with an expected mean $>>2N_e\mu$ differences per site (Birky et al., 2010). The pairwise sequence differences were calculated in MEGA 5.0 (Tamura et al., 2011), using the concatenated dataset.

2.5. Detection of recombination

The criterion of incongruence among the four gene genealogies was used to infer the occurrence of sexual recombination among isolates, using the Phi-test implemented in SplitsTree (Huson, 1998), which uses the pairwise homoplasy index, PHI ($=\Phi$) statistic, to detect refined incompatibility indicating recombination (Bruen et al., 2006). In selected cases, also the IA (Index of Association) test, which measures whether the alleles from different loci in a population are randomly or non-randomly associated in the analyzed genomes (Maynard Smith, 1992) was used. The latter method was computed by Multilocus 1.3.b (Agapow and Burt, 2001).

3. Results

3.1. Sample design and phylogenetic markers

The sample (Table 1) consisted of 112 strains, and included strains of putatively new and previously recognized species of the *Longibrachiatum* clade (Samuels et al., 1998, Mullaw et al., 2010; Druzhinina et al., 2008, 2010) and strains that were attributed to this group based on their morphology and/or DNA sequences using *Tricho*BLAST (for *tef1* and *rpb2)* and ITS1 and 2 in *TrichO*Key (Druzhinina et al., 2005; Kopchinskiy et al., 2005) as implemented on www.isth.info or BLAST on the NCBI portal http://blast.ncbi.nlm.nih.gov/. Many of these strains have previously been reported in the literature (Wuczkowski et al., 2003; Bissett et al., 2003; Kubicek et al., 2003; Druzhinina et al., 2005, Druzhinina et al., 2008, 2010; Atanasova et al., 2010; Mullaw et al., 2010). Where possible, strains with the same ITS1 and 2 allele were selected from diverse regions to cover the maximum of geographic distribution.

Individual nucleotide characteristics of the loci are shown in Table 2. Tajima's D test confirmed neutral evolution for all four gene fragments. No conflict was detected between loci and the bivariate plot of bipartitions for the Bayesian analyses suggested convergence between parallel runs.

Table 2
Nucleotide parameters of loci used for phylogenetic analysis.

	tef1	*cal1*	*chi18-5*	Total
Total sites	522	429	704	1655
Sites without gaps	229	179	526	934
Parsimony informative sites	45	53	145	243
nt diversity π	0.0562	0.0261	0.0711	
Tajima's D	NS[a]	NS	NS	

[a] NS, not significant, $P < 0.01$.

3.2. Molecular phylogeny

We first used Bayesian methods to infer genealogies from three single locus alignments. The trees were rooted against *T. virens* (teleomorph *H. virens*), which formed a basal branch to *T. reesei*, a member of the *Longibrachiatum* clade, in a genome-wide phylogeny (Kubicek et al., 2011). Phylograms obtained from *tef1*, *chi18-5* and *cal1* had a well-resolved internal structure with supported internal nodes (Supplementary data 1), while *rpb2* resulted in a poor phylogenetic resolution and therefore was excluded from the subsequent analysis (data not shown). Fifteen terminal phylogenetic clades with posterior probabilities >0.94 and eleven lone lineages were consistently observed in all three trees. Therefore, based on the strict criteria of GCPSR, they can be considered as phylogenetic species. To prove the genealogical concordance of these clades, we used the approach of Dettman et al. (2003) and analyzed a majority-rule consensus tree from the three single-locus trees (Supplementary data 2), which approved these clades. Because of the congruence of the gene trees, we ran a Bayesian analysis with a concatenated dataset of the three genes (Fig. 1). Eleven clades and the lone lineages of *T. effusum* C.P.K. 254 (Bissett et al., 2003) and *H. andinensis* G.J.S. 90-140 contained type strains of formally established taxa (Table 1, Fig. 1). They are indicated by an arrow on the branch leading to the respective node on the concatenated phylograms (Fig. 1). Two clades (*T.* sp. PS III and *T.* sp. C.P.K. 3334) and the lone lineages *H.* sp. CBS 243.63 have been previously considered as putative new species awaiting formal taxonomic description (Druzhinina et al., 2008; Atanasova et al., 2010; Mullaw et al., 2010). They are indicated by a double arrow respectively. The three other clades (*H.* sp. nov. G.J.S. 02-120, *T.* sp. nov. TR175 and *T.* sp. nov. MA 3642), four lone lineages (isolates G.J.S. 08-81, G.J.S. 00-72, G.J.S. 99-17 and G.J.S. 10-263), and a group of strains (C.P.K. 667, G.J.S. 09-62, G.J.S. 01-355) affiliated with the type strain of *H. andinensis* (G.J.S. 90-140) could not be attributed to any known species by GCPSR concept.

3.3. Species recognition

Phylogenetic analysis using GCPSR supported a monophyletic origin of the *Longibrachiatum* clade and all of the species that were recognized by Bissett in 1984 and later authors (Doi et al., 1987; Samuels et al., 1998; Bissett, 1991; Bissett et al., 2003; Atanasova et al., 2010). In order to test whether the additional clades or lone lineages that have not been formally described may represent putatively species, we measured their phylogenetic distance from the neighboring clades by the K/θ method (Birky et al., 2010). As can be seen in Table 3, all of the previously

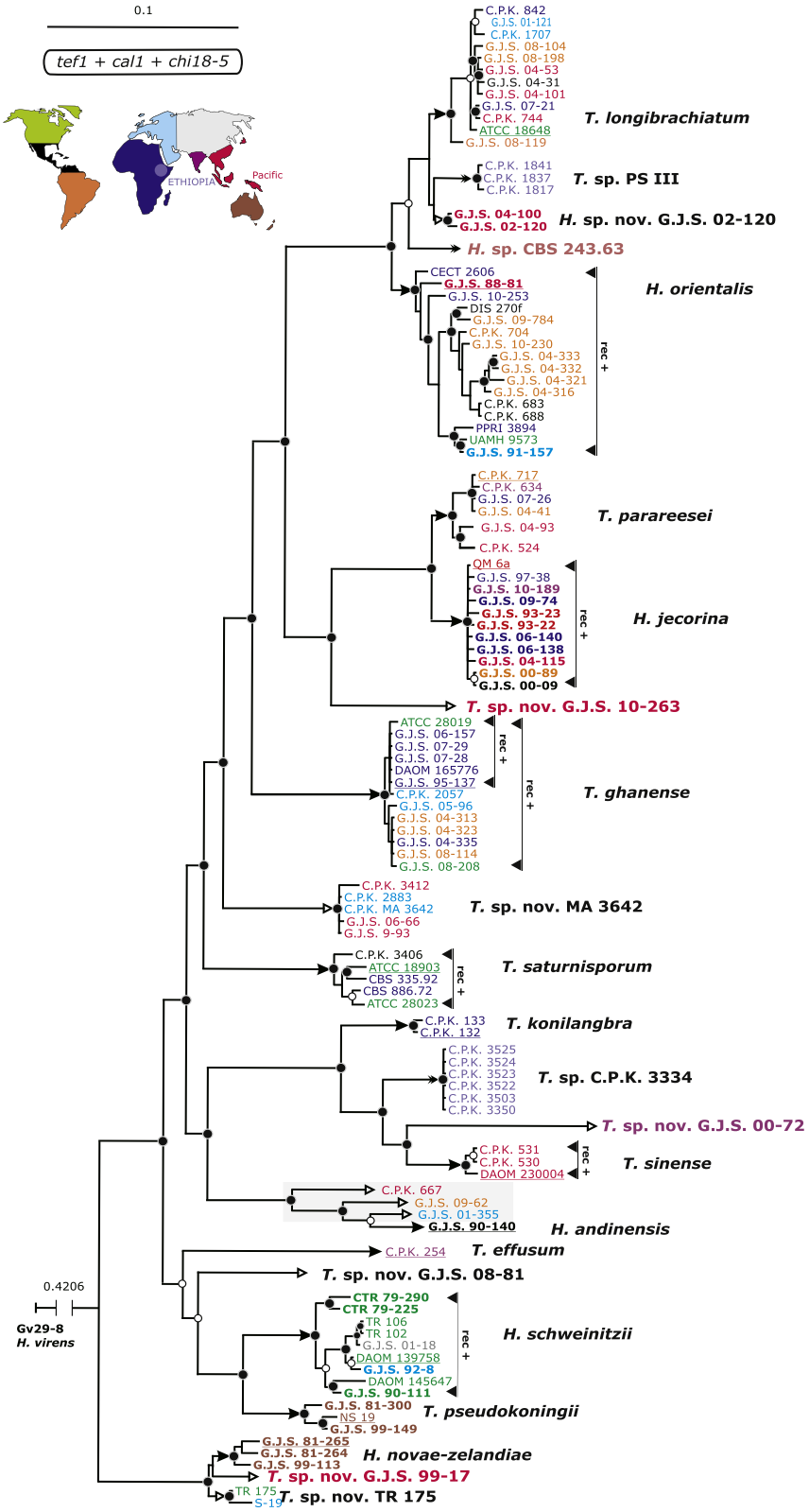

Fig. 1. Bayesian phylograms obtained from the concatenated alignment of *tef1*, *cal1* and *chi18-5* loci. Branches leading to formally described or previously recognized phylogenetic species are marked by filled single and double arrows respectively; phylogenetic species recognized in this study are shown by open arrows. The color code corresponds to the map insert and indicates geographic origin of isolates. Nodes supported by posterior probability >0.94 are shown in circles: black circles indicate supports obtained in both analyses after removal of ambiguous areas of the alignment using unconstrained gblocks (Castresana, 2000) and without such treatment, while white circles indicate supports obtained based on the complete concatenated alignment only. Sexual recombination is shown by vertical bars with a 'rec+' sign. Type strains of formally described species are underlined. Strains isolated from teleomorphs are given in bold.

known species were supported by values of >4, and this also turned out to be true for all but one of the new clades of unknown species identity. The only exception was the clade containing *H. novae-zelandiae*, where the branch to *T.* sp. TR175 was not supported. Also, the hypothesis that *T. ghanense* would consist of two cryptic species received no support.

As inferred from Birky et al. (2010), the species identified by the 4× rule could theoretically also be metapopulations that consist of two or more local populations connected by migration or by periodic extinction and re-colonization. An additional problem, particularly relevant in this case, are the lone lineages for which no π or θ could be determined and therefore their species status could not be clarified. To solve such cases, Birky et al. (2010) introduced a further criterion to distinguish between species and populations of species by plotting the sequence differences versus the respective number of pairs of strains. As already shown by Highton (2000), this will result in a bimodal frequency distribution of pairwise sequence differences, among which the lower values represent sequence differences between individuals within species, with an expected mean of $2N_e\mu$ differences per site, whereas the second mode represents differences between species with an expected mean $\gg 2N_e\mu$ differences per site (Birky et al., 2010). We therefore plotted the sequence differences in *tef1*, *cal1* and *chi18-5* of the investigated 104 isolates versus the nucleotide differences of all pairs (Fig. 2). As can be seen, this resulted in a bimodal distribution, although the distribution in the second mode (which represents higher diversities) was not perfectly bell shaped. Nevertheless, the first mode (supposed to represent the sequence diversity between individuals within species occurred at diversities of 0–0.01. This fits nicely to the mean $2N_e\mu = \theta$ value of 0.00754 calculated for our sample. Hence we consider values >0.02 to be indicative of differences between species, which is supported by the fact that 0.02 already lies within the onset of the second mode.

All of the species that were detected by the 4× rule showed values >0.02 when compared with other species. All clades identified above were approved by pairwise sequence differences of their isolates with those from other clades of >0.02, whereas intraspecific pairwise differences were always <0.02. This method also showed that the sister species to *H. novae-zelandiae*, *T.* sp. nov. are separate species (pairwise sequence difference 0.028).

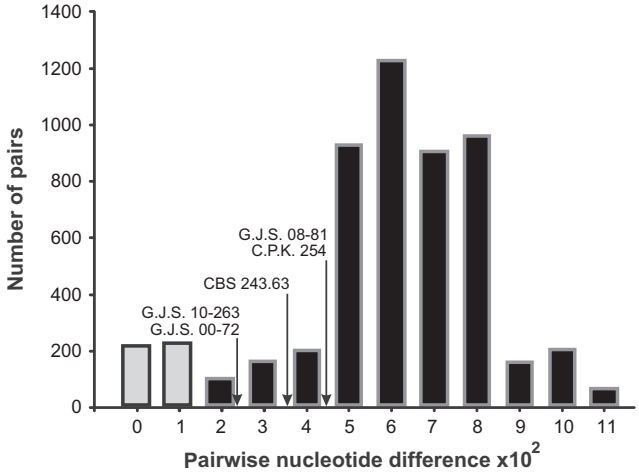

Fig. 2. Frequency distribution of uncorrected pairwise sequence differences, in bins of 1%. The gray bars show values detected between isolates of the same species. Arrows with strain numbers identify the lowest difference value detected for this isolate against isolates from any other taxon.

Consequently, we also tested the nucleotide differences between the lone lineages and other isolates (Fig. 2). This analysis confirmed G.J.S. 10-263, G.J.S. 08-81, G.J.S. 00-72, G.J.S. 99-17, CBS 243.63 and C.P.K. 254 (*T. effusum*) to be individual species, but did not support it for C.P.K. 524 and G.J.S. 04-93. These therefore should be attributed to *T. parareesei*.

3.4. Evolution of phenotypical traits

There is a high degree of phenotypic consistency among the members of the *Longibrachiatum* clade, which is reflected e.g. in a strong tendency for species to be thermotolerant (i.e. they still grow at 37 or 40 °C), and that the morphology of the conidiophores and conidia is largely homogeneous and in agreement with earlier descriptions by Bissett (1984). Thus it is not surprising that the main clades that are supported by GCPSR and the K/θ method, show only subtle internal phenotypic variation, and differences

Table 3
Pairwise calculations of 4× rule for clades recognized based on genealogical concordance.

Species	Next neighbor	θ	K	K/θ
T. sp. G.J.S. 10-263	*T. reesei*	0.00127[a]	0.045	**35.7**[b]
T. parareesei	*T. reesei*	0.00199	0.176	**88.4**
H. sp. G.J.S. 02-120	*T.* sp. PS III	0.00234	0.177	**75.7**
H. sp. CBS 243.63	*H. orientalis*	0.00127[a]	0.180	**141.7**
H. sp. G.J.S. 02-120	*T. longibrachiatum*	0.00234	0.124	**53.0**
H. sp. CBS 243.63	*H.* sp. G.J.S. 02-120	0.00127[a]	0.078	**61.41**
T. ghanense type subclade	*T. ghanense* none type subclade	0.00485	0.004	0.8
T. ghanense none type subclade	*T. ghanense* type subclade	0.00056	0.004	**7.3**
T. sp. MA 3642	*T. ghanense*	0.00238	0.010	**43.7**
T. saturnisporum	*T.* sp. MA 3642	0.01375	0.089	**70.1**
T. sinense	*T.* sp. G.J.S. 00-72	0.00403	0.035	**8.6**
T. konilangbra	*T. sinense*	0.00154	0.021	**13.8**
T. sp. C.P.K. 3334	*T. konilangbra*	0.00127[a]	0.195	**153.2**
T. sp. C.P.K. 3334	*T. sinense*	0.00127[a]	0.042	**33.1**
T. effusum	*H. schweinitzii*	0.00127[a]	0.230	**181.1**
T. effusum	*T.* sp. G.J.S. 08-81	0.00127[a]	0.087	**68.5**
T. sp. G.J.S. 08-81	*H. schweinitzii*	0.00127[a]	0.148	**116.5**
H. schweinitzii	*T. pseudokoningii*	0.01389	0.062	**4.5**
H. novae-zelandiae	*T. saturniopsis*	0.01006	0.01736	1.71
H. novae-zelandiae	*T.* sp. G.J.S. 99-17	0.01006	0.01616	1.58
T. sp. S 19	*T.* sp. G.J.S. 99-17	0.00965	0.02234	2.31

[a] θ was calculated based on a single strain, see Section 2.4 for details.
[b] Bold font highlights values >4.

Table 4
Recombination and evolution of species from the *Longibrachiatum* clade.

	n	Tajima's *D*	Fu and Li's *D*	*Φ*-test	IA test[c]
H. andinensis	4	−0.494	−0.436	0.3029	0.86[c]
T. sp. MA 3642	5	−1.161	−1.167	NA[a]	NA
T. sp. PS III	3	NP[b]	NP	0.223	0.96
T. sp. C.P.K. 3334	6	NP	NP	NA	NA
T. ghanense					
Subclade with the type strains	8	−0.509	−1.168	0.0361	0.37
All strains		−0.722	−0.933		0.41
Subclade without the type strain	5	−0.972	−0.972	0.233	1.335[c]
H. jecorina	11	−1.256	−1.256	0.006	0.175
T. konilangbra	2	NP	NP	NA	NA
T. longibrachiatum	12	−1.7	−2.025	0.58	0.88
H. novae-zelandiae	3	NP	NP	0.36	0.79[c]
H. orientalis	17	−0.164	0.087	0.0005	0.12
T. parareesei	4	−0.212	−0.212	0.126	0.93
H. sp. G.J.S. 02-120	2	NP	NP	NA	NA
H. pseudokoningii	3	NP	NP	NA	NA
T. saturnisporum	5	−0.641	−0.573	0.0313	0.42
T. sinense	3	NP	NP	0.02	NA
H. schweinitzii	9	−0.537	−0.561	0.00058	0.22

[a] NA, not analysed; species or putative species known only from a single isolate were not included.
[b] NP, not possible: calculation not done because of insufficiently large sample.
[c] In these cases p was > 0.05, and the data are thus questionable.

are mainly reflected in dimensions of conidia or rates of growth. There are, however, notable exceptions: *Hypocrea novae-zelandiae* is apparently endemic to New Zealand, where it has been collected as teleomorph. Its *Trichoderma* anamorph is unremarkable in the *Longibrachiatum* clade. Most species in the *Longibrachiatum* clade have smooth, ellipsoidal to oblong conidia, but conidia of *T. ghanense*, *T. saturnisporum* and *T.* sp. TR 175 are typically tuberculate to a greater or lesser degree. The basal position of the *H. novae-zelandiae* clade in Fig. 1 may indicate that tuberculate conidia, which are also found in the Viride clade (Jaklitsch et al., 2006) may be an ancestral trait of the *Longibrachiatum* clade. *Trichoderma effusum* and the phylogenetic species *T.* sp. G.J.S. 08-81 respectively, are phenotypically divergent (Samuels et al., in press) to such an extent that, based on their morphology alone, they would not have been considered as members of the clade.

Stromata of most members of the *Longibrachiatum* clade are brown but in one single subclade, which includes the sexually reproducing species *H. schweinitzii* (anamorph *T. citrinoviride*) and *H. pseudokoningii*, stromata are black or nearly so.

3.5. Sexual recombination

We have previously reported that closely related species of the *Longibrachiatum* clade can survive based on alternative (combined sexual and asexual or exclusively asexual) reproduction strategies (Druzhinina et al., 2008, 2010). In order to identify clonal and mainly sexually recombining species in the whole clade, we used the Phi-test built on the pairwise homoplasy index (PHI, *Φ*) to detect refined incompatibility even in the presence of recurrent mutation (Bruen et al., 2006). This method assumes the infinite sites model of evolution, in which the detection of incompatibility for a pair of sites indicates recombination. It detected recombination within *H. schweinitzii/T. citrinoviride*, *T. sinense, T. saturnisporum, H. jecorina/T. reesei, H. orientalis* and the subclade within *T. ghanense* that contains the type strain G.J.S. 95-137 (Table 4), but not in any of the other clades shown in Fig. 1. Recombination was also evident from the topology of single locus trees, which showed incongruent positions of individual isolates within these species (cf. Supplementary data 1). Interestingly, no recombination was detected between strains basal to the type strains of either *H. andinensis* or *H. novae-zelandiae*, which both were isolated from their teleomorphs.

In addition, we tested these strains by the index of association test, which confirmed all the recombining taxa that were identified by the PHI-test (Table 4).

4. Discussion

In the present paper, we extended and complemented the well-known GCPSR concept for species delimitation by the addition of the population genetics-based K/θ method to identify species within the *Longibrachiatum* clade of *Hypocrea/Trichoderma*. We show that the results obtained by the two methods agree with each other well, and detected 26 phylogenetic species which is more than a doubling of the species inventory already known for this group (Samuels et al., 1998). All of the previously described taxa and all but one of the proposed phylogenetic species were confirmed. In addition, two new phylogenetic species were identified.

However, we also noted some problematic cases: the four isolates that according to GCPSR represent *H. novae-zelandiae* and the two isolates that represent *T.* sp. nov. TR 175, respectively, were not supported by the K/θ method. This finding was particularly puzzling in view of the fact that they have already acquired a number of distinct phenotypical characters that would be consistent with their nature as a separate species. Also, their pairwise nucleotide differences (0.026–0.032) placed them into the mode typical for separate species (cf. Fig. 2). Birky et al. (2010) observed a similar case in some *Penicillium* clades. It is possible that in the present case, the failure to pass the K/θ method is either due to a still incomplete sampling of the genetic diversity of *H. novae-zelandiae* and phylogenetic species *T.* sp. nov. TR 175, or, less likely, to an unusually high plasticity of phenotypic characters. If two, recently diverged clades are now genetically isolated but share retained ancestral variation, their divergence and genetic isolation follow a continuum and no single percentage is going to work in all cases. Thus the K/θ method will not work in such cases.

Another interesting case was the branch containing the type strain of *H. andinesis.* Based on the principles of GCPSR and the strong statistic support for this branch, all isolates in this clade would be identified as the same species. Yet, it was suspicious that the genetic distances between these isolates were much greater than those observed among isolates of other species within the *Longibrachiatum* clade. A calculation of the pairwise sequence differences between the four isolates of the *H. andinensis* clade revealed

values between 0.028 and 0.062 what corresponds to different species in the sense of our sample. In addition, tests for recombination within this clade frequently gave negative result what contrasts with the findings that the type strain of *H. andinensis* (G.J.S. 90-140) was sampled as a teleomorph. We therefore consider these strains to represent closely related but rare species, for which we find it practicable to continue calling these strains "*H. andinensis* complex" until more isolates of them have been found.

It was also conspicuous that 25% of the identified phylogenetic species were represented only by a single isolate, i.e. they formed lone lineages. All of them exhibited a basal position to the species clusters they were associated with, and were characterized by long genetic distances and nucleotide sequence diversities >0.025, thus implying an already long history of existence as a separate species. Theoretically, they could be species with growth requirements that exceed those fulfilled by the media used for isolation of *Trichoderma* from the environment; yet none of these has so far yet been detected in metagenomic studies on *Trichoderma* (Hagn et al., 2007; Friedl and Druzhinina, 2012). Alternatively it is possible that they represent relict species that are in progress of extinction. However, the most likely interpretation is that these species are strongly biased in their habitat and geographic distribution and have therefore not been found so far. Similar cases were observed for the genetically diverse *Harzianum* clade of *Trichoderma* by Druzhinina et al. (2010).

Six of ten species of the *Longibrachiatum* clade, for which enough isolates were available to test for a history of recombination, were shown to exhibit evidence for sexual recombination. With the exception of *H. andinensis* and *H. novae-zelandiae* (which was above explained as a sampling problem), all of the species for which also sexual stages were sampled, in fact confirmed recombination, thereby also verifying the validity of our approach. In addition, two species for which so far no teleomorph has been found (*T. ghanense, T. saturnisporum*) were also positive in this test. An interesting finding from the recombination tests was that there are some phylogenetic clades in *Longibrachiatum*, which contain a sexual and an apparently asexual species (e.g. *T. longibrachiatum* versus *H. orientalis*; *T. reesei* versus *T. parareesei*; *T. sinense* versus *T.* sp. nov. C.P.K. 3334), suggesting that speciation in these cases involved loss or gain of sexual reproduction. This phenomenon is also seen in *T. ghanense*, which was shown to split into two phylogenetic groups: the clade containing the type strain showed a history of recombination, the other clade did not. It is possible that the latter clade represents a species in progress. One must apply caution to these analyses, however, because undetected population structure, or lack of sufficient variation among the individuals may obscure the detection of recombination. So the inability to detect recombination does not necessarily equate with asexuality.

Summarizing, our data show that the combination of GCPSR with the K/θ method represents a robust test to identify phylogenetic species in fungi. Although Birky et al. (2010) propose the K/θ method only for asexual fungi, our current results demonstrate that this approach is also applicable to a phylogenetic analysis of fungi which consist of a mixed batch of sexual and asexual taxa. In addition, combining the K/θ method with GCPSR helped to deepen the analysis and to exclude false positives. We therefore recommend combining these two methods also in future studies with other fungi.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2012.02.004.

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